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CSK HOMOLOGONS KINAZE (CHK) FOR DETECTING AND TREATING BREAST CANCER

Abstract:

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(A1) Methods using CSK homologons kinaze (CHK) for detecting and treating breast cancer are described.

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1 PTK 1/3 PRIMERS 21 41
5' ggatccattcacagagacctagcagcacgcaacatcctggtctcagaggacctggtaacc
G S I H R D L A A R N I L V S E D L V T

61 81 101
aaggtcagcgactttggcctggccaaagccgagcggaaggggctagactcaagccggctg
K V S D F G L A K A E R K G L D S S R L

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(57) Abstract

Methods using CSK homologons kinaze (CHK) for detecting and treating breast cancer are described.

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CSK HOMOLOGONS KINAZE (CHK) FOR DETECTING AND TREATING BREAST CANCER

RELATED APPLICATIONS

This application is a continuation-in-part of pending U.S. Serial No. 08/876,882, filed June 16, 1997, which claims the benefit of Provisional Application No. 60/035,228, filed January 8, 1997, the contents of which are herein incorporated by reference in their entirety.

GOVERNMENT SUPPORT

This invention described herein was supported in whole or part by the National Institutes of Health Grant Nos. HL51456-02 and HL51456. The United States Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Breast cancer is the second leading cause of cancer death among women in the United States and is the leading cause of death among women aged 30-70. (Abeloff, M.D., *Curr. Opin. Oncol.*, 8:447-448 (1996)). The inheritance of germ-line mutations in autosomal dominant susceptibility genes appears to be responsible for 5-10% of all breast cancer cases (Fitzgerald, M.G., *et al.*, *New Engl. J. Med.*, 334:143-149 (1996)), and up to 36% of the cases diagnosed before age 30. BRCA1 was the first isolated breast cancer susceptibility gene (Langston, A.A., *et al.*, *New Engl. J. Med.*, 334:137-142 (1996); Couch, F.J. and Weber, B.L., *Hum. Mutat.*, 8:8-18 (1996)) and mutations in BRCA1 alone account for approximately 45% of the families with high incidence of breast and ovarian cancer (Chen, Y.M., *et al.*, *Science*, 272:125-126 (1996); Sully, R., *et al.*, *Science*, 272:123-126 (1996)). In addition, a second breast cancer susceptibility gene, BRCA2, has been isolated recently

(Wooster, R., *et al.*, *Nature*, 378:789-792 (1995); Tavtigian, S.V., *et al.*, *Nat. Genet.*, 12:333-337 (1996)).

However, the majority of breast carcinomas appear to be sporadic and have a complex accumulation of molecular and cellular abnormalities that constitute the malignant phenotype. A number of somatic gene alterations, such as loss of expression of specific tumor suppressor genes, have been found to occur in primary human breast tumors (Borg, A., *et al.*, *Cancer Res.*, 52:2991-2994 (1992); Eeles, R.A., *et al.*, *Cancer Surveys*, 25:101-124 (1995)). Additionally, there is considerable evidence that genetic alterations in growth factor signaling pathways can contribute to human breast malignancies. In this regard, activation of different proto-oncogenes has been found in primary breast tumor (Berns, E.M., *et al.*, *Cancer Res.*, 52:1036-1039 (1992); Borg, A., *et al.*, *Brit. J. Cancer*, 63:136-142 (1991); Gullick, W.J., *et al.*, *Brit. J. Cancer*, 63:434-438 (1991)). Thus, there is considerable importance in identifying, at a molecular level, factors that contribute to the progression from normal growth towards malignancy.

SUMMARY OF THE INVENTION

The present invention relates to the demonstration that a cytoplasmic protein tyrosine kinase, Csk Homologous Kinase or CHK, is expressed in human breast cancer, but not in adjacent normal breast tissue. Specifically, the present invention relates to methods of detecting the presence of cancer in mammalian breast tissue by the detection of the protein tyrosine kinase CHK, or the detection of nucleic acids encoding the CHK in mammalian tissue, specifically breast tissue. The detection of CHK in breast tissue is indicative of cancer.

The presence of CHK in breast tissue can be determined by detecting the expression of CHK protein, or a protein fragment, in breast tissue samples obtained from the mammal. Typically, an agent, such as an antibody, is used to detect expressed CHK protein. For example, biopsy tissue can be obtained from the mammal, fixed in a suitable

medium and contacted with anti-CHK antibodies, for example rabbit anti-CHK, which specifically bind to the CHK protein if it is present in the tissue sample. The anti-CHK antibody can itself be detectably labeled, or a detectably labeled second antibody, for example, peroxidase-conjugated mouse anti-rabbit antibody, can be used.

The presence of CHK in breast tissue can also be detected using an immunoblot (e.g., Northern blot) assay. For example, tissue can be obtained from the mammal and a cell lysate prepared which contains proteins released from the tissue cells. The lysate proteins can be separated by electrophoretic means, such as by size via SDS polyacrylamide gel electrophoresis, and contacted with anti-CHK antibody which specifically binds to CHK if it is present in the lysate. Again, the anti-CHK antibody can be detectably labeled, or a detectably labeled second antibody can be used. Alternatively, CHK protein present in a cell lysate can be detected by enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) or other immunoassays.

The presence of CHK in breast tissue can also be determined by detecting the presence of a nucleic acid sequence encoding all, or a portion, of the CHK protein. The nucleic acid can be DNA or RNA. For example, genomic DNA, cDNA or RNA can be obtained from a sample of breast tissue and contacted with an agent such as a polynucleotide probe that forms a stable hybrid with the nucleic acid sequence encoding CHK. The probe can be detectably labeled. The DNA or RNA obtained from the mammal can be amplified prior to assay, for example using the polymerase chain reaction (PCR) or the ligase chain reaction (LCR), using specific nucleic acid primers. Primers useful to amplify the CHK nucleic acid specifically hybridize to the CHK nucleic acid or to nucleic acid sequence that flanks the target CHK nucleic acid sequence region.

Overexpression of the receptor tyrosine kinase, ErbB-2 (also termed *neu*/HER-2) has been associated with the development of breast cancer. (Slamon, D.J., *et al.*, *Science*, 244:707-712 (1989); Olsson, H., *et al.*, *J. Natl.*

Cancer Instit., 83:1483-1487 (1991)). A common pathway linking the activation mechanisms in ErbB-2 amplification in breast cancer is increased tyrosine kinase activity which leads to cellular transformation. The abundance of ErbB-2 receptors and their ligands (e.g., heregulin or HGR) in breast cancer points to a functional role in the pathogenesis of this malignancy. As demonstrated herein, CHK specifically interacts with activated ErbB-2 upon HGR stimulation and results described herein suggest that CHK functions as a negative regulator of ErbB-2 mediated mitogenic signaling.

Accordingly, the present invention also encompasses methods of inhibiting breast cancer cell growth (also referred to herein as neoplastic cell growth), specifically ErbB-2 mediated neoplastic cell growth, by supplying CHK to cancer cells. As used herein, the term "CHK" encompasses the CHK protein, CHK peptides with biological activity, CHK analogs and CHK derivatives with biological activity. For example, CHK protein, peptide or a biologically active fragment thereof, or a CHK analog or derivative, can be supplied to mammalian breast tissue which is abnormal, e.g., neoplastic, or at risk of becoming abnormal. The CHK protein, peptide, analog or derivative binds ErbB-2 and inhibits, or propholactically prevents cancer cell growth. The CHK protein or peptide can be supplied to the target breast tissue by introducing into target cells a liposome preparation that contains CHK. Specifically encompassed by this invention is the topical application of such liposomes in a cream or ointment.

Alternatively, CHK can be supplied to the target tissue by introducing a nucleic acid sequence encoding CHK, or a biologically active fragment, analog, or derivative of CHK which is then expressed in the breast tissue.

As described herein, for the first time, Csk-homologous Kinase has been identified as playing an important role in signaling in neoplastic breast tissue and as functioning as a negative regulator of ErbB-2. As a

result of this work, novel methods of detecting and inhibiting breast cancer are now available.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A to 1D are a series of four autoradiographs showing the association of the CHK-SH2 domain with ErbB-2 upon stimulation with HRG. Lysates were precipitated with the CHK-SH2 GST fusion protein and immunoblotted with either monoclonal antiphosphotyrosine antibody (PY20), (Fig. 1A), or with polyclonal anti-ErbB-2 antibodies (Fig. 1B). Immunoprecipitations of the same lysates were performed using 3E8 monoclonal anti-ErbB-2 antibody and blotted with PY20 (Fig. 1C) or with anti-ErbB-2 antibodies (Fig. 1D). Molecular size markers are indicated on the left (kDa).

Figure 2 shows the nucleotide sequence (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of two overlapping *matk* cDNA clones representing the full-length cDNA. Nucleotide numbers are shown on the left. The putative initiation codon at nucleotide position 263 is shown in bold type.

Figure 3 shows the nucleotide sequence (SEQ ID NO: 3) and deduced amino acid sequence (SEQ ID NO: 4) of a CHK fragment. Specific primer positions are indicated.

Figures 4A, 4B and 4C are graphs showing suppression of cell growth by CHK. Figures 4A and 4B are graphs showing the mitogenic effects of heregulin on MCF-7 clones grown without (4A) or with 10 nM heregulin for 24 or 48 hours (4B). Each data point is the mean of three readings within the same experiment. Each graph is one representative experiment out of three. Figure 4C is a bar graph showing numbers of colonies formed in soft agar by MCF-7 clones.

Figure 5 is a bar graph showing immune complex kinase reactions. MCF-7 cells were infected with either a CHK-vaccinia recombinant virus and T7 polymerase virus ("CHK") or with T7 virus alone as a control ("T7"), and either

stimulated with 10 nM heregulin ("+HRG") or left as controls ("-HRG").

Figure 6 is a bar graph showing results of an *in vitro* kinase assay of CHK and Csk immunoprecipitates. Mouse brain extracts were immunoprecipitated with anti-CHK, anti-Csk, or normal mouse serum. These immunoprecipitates were then used to phosphorylate either Poly Glu/Tyr (□), enolase (■), or C-terminal *src* peptide (▒).

DETAILED DESCRIPTION OF THE INVENTION

The family of protein tyrosine kinases (PTKs) includes oncogenes and growth factor receptors, several of which have been linked to the pathogenesis and progression of certain cancers (Bishop, J.M., *Genes. Dev.*, 9:1309-1315 (1995) Cance, W.G., *et al.*, *Breast Can. Res. & Treat.*, 35:105-114 (1995)). Increasing evidence indicates that the c-*src* proto-oncogene may play an important role in breast cancer. Human breast cancers often show much higher levels of *src* protein kinase activity than normal adjacent epithelium (Hennipman, A., *et al.*, *Cancer Research*, 49:516-521 (1989), Ottenhoff-Kalff, A.E., *et al.*, *Cancer Research*, 52:4773-4778 (1992)). Indeed, about 70% of the elevated total tyrosine kinase activity found in primary breast cancers can be attributed to increased *src* activity. Involvement of pp60c-*src* with two major signaling pathways in human breast cancer has been demonstrated. In human breast carcinoma cell lines, the SH2 domain of *src* binds to activated epidermal growth factor (EGF-R) and $p185^{ErbB-2}$, a receptor tyrosine kinase (Luttrell, D.K., *et al.*, *Proc. Natl. Acad. Sci. USA*, 91:83-87 (1994)).

Overexpression of the receptor tyrosine kinase ErbB-2 (also termed *neu*/HER-2) has been also associated with the development of breast cancer (Salmon, D.J., *et al.*, *Science*, 244:707-712 (1989), Williams, T.M., *et al.*, *Pathobiology*, 59:45-52 (1991)). A common pathway linking the activation mechanisms in ErbB-2 amplification in breast cancer is increased tyrosine kinase activity which leads to

cellular transformation (Olsson, H., *et al.*, *J. Natl. Cancer. Inst.*, 83:1483-1487 (1991)).

Four members of the ErbB (HER) family are presently known: $p170^{ErbB-1}$ (epidermal growth factor receptor, EGR-R), $p185^{ErbB-2}$, $p180^{ErbB-3}$ and $p180^{ErbB-4}$. In particular, the overexpression of the $p185^{ErbB-2}$ correlates with a poor clinical prognosis in breast cancer (Beerli, R.R., *et al.*, *Mol. Cell. Biol.*, 15:6496-6505 (1995), Holmes, W.E., *et al.*, *Science*, 256:1205-1210 (1992), Wen, D., *et al.*, *Cell*, 69:559-572 (1992)). The overall amino acid homology within this receptor family ranges from 40-50%, and all the family members are characterized by two cysteine-rich regions in the extracellular domain, a single transmembrane region and a large cytoplasmic domain that exhibits tyrosine kinase activity (Wen, D., *et al.*, *Cell*, 69:559-572 (1992)).

Several ligands that bind to and stimulate the kinase activity of the ErbB family members have been identified and are classified as EGF-like ligands. EGF, HB-EGF, amphiregulin, betacellulin, epiregulin and transforming growth factor-α (TGF-α) are the ligands for the EGF-R (ErbB-1) (Cohen, B.D., *et al.*, *J. Biol. Chem.*, 271:4813-4818 (1996), Johnson, G.R., *et al.*, *J. Biol. Chem.*, 268:2924-2931 (1993)). Heregulin (HRG) and its rat homologue, *neu* differentiation factor (NDF), are a subfamily of neuregulins which are EGF-like ligands that bind to and activate both ErbB-3 and ErbB-4. Although none of these factors binds directly to the ErbB-2, both EGF and HRG induce its tyrosine phosphorylation, presumably by ligand-driven heterodimerization and cross-phosphorylation. Interestingly, ErbB-2, by heterodimerizing with the EGF-R and ErbB-3, confers high affinity binding sites for EGF and HRG, respectively (Beerli, R.R., *et al.*, *Mol. Cell. Biol.*, 15:6469-6505 (1995), Marchionni, M.A., *et al.*, *Nature*, 362:312-318 (1993)).

Recently, a cytoplasmic tyrosine kinase, CHK (Csk Homologous Kinase), previously referred to as MATK (Megakaryocyte Associate Tyrosine Kinase), has been identified. The CHK protein, primarily expressed in

hematopoietic cells and in human brain has an apparent molecular weight of 58 kDa, and shares 50% homology with the human Csk (c-terminal *src* kinase). Like Csk, CHK contains SH3, SH2 and tyrosine kinase domains, and lacks the *src* family N-terminal myristylation and autophosphorylation sites. CHK was found to phosphorylate the inhibitory carboxyl-terminal conserved tyrosine of several src-related enzymes *in vitro*, including Lck, Fyn and c-src, and to reduce the elevated phosphotyrosine levels of *src* family kinases in Csk-deficient fibroblasts.

As described herein, for the first time, the interaction of CHK with ErbB-2 upon the activation of breast cancer cells by HRG has been demonstrated. This interaction occurred via the SH2 domain of CHK and was specific to the activated ErbB-2 receptor upon HRG stimulation. Also described herein for the first time, is the demonstration that CHK is expressed in human breast cancer cells but not in adjacent normal breast tissue cells.

The present invention relates to methods of detecting and treating breast cancer in mammals wherein the methods encompass the detection or use of CHK protein, or nucleic acids encoding CHK. As defined herein, the term "CHK protein" encompasses the full-length CHK protein as described in Bennett, B.D., *et al.*, *J. Biol. Chem.*, 269:1068-1074 (1995) and also biologically active CHK fragments (e.g., biologically active peptides), derivatives analogs, variants and mutants.

The term "biologically active" CHK fragments, peptides, derivatives, analogs, variants and mutants is defined herein as fragments, peptides, derivatives, analogs variants and mutanats with biological activity encompassing the specific association of CHK with the intracellular domain of ErbB-2, or chimeric ErbB-2 molecules, such as EGF-ErbB-2 molecules. Encompassed is the binding of CHK via its SH2 domain to ErbB-2. Specifically, encompassed herein is the binding of CHK Tyr^{1253} of ErbB-2 as described herein. As described herein, this association is mediated

by the SH2 domain of CHK. Association of CHK with ErbB-2 can be demonstrated, for example, using immunoprecipitation experiments as described in the Examples. Because CHK is a tyrosine kinase, biological activity is also defined herein as the ability of CHK to phosphorylate tyrosine, specifically the phosphorylation of the carboxyl-terminal tyrosine of src-related kinases, thereby repressing their activity. Several src-related kinases include Lck, Fyn and c-src. Assays that demonstrate the phosphorylation ability of CHK include immune complex kinase reactions and the ability to phosphorylate kinases in yeast co-expression systems as described in Avraham, S. *et al.*, *J. Biol. Chem.*, 270:1833-1842 (1995), Chow, L.M. *et al.*, *Oncogene*, 9:3371-3374 (1994), Klags, S. *et al.*, *Proc. Natl. Acad.* Sci. USA, 19:2597-2601 (1994) and Davidson, D. *et al.*, *J. Biol. Chem.*, 272:1355-1362 (1997). Other methods of measuring kinase activity are known to those of skill in the art.

Another biological activity of CHK is the antigenic property of CHK binding specifically to anti-CHK antibody (e.g., antigenicity). Also encompassed is the activity of CHK to elicit inducing a specific immunological response (e.g., immunogenicity) as determined using well-known laboratory techniques. For example, biologically active CHK can induce an immunological response which produces antibodies specific for CHK (anti-CHK antibodies).

To be "functional" or "biologically active," a CHK protein fragment, peptide, analog, mutant or derivative typically shares substantial sequence (amino acid or nucleic acid) identity (e.g., at least about 65%, typically at least about 80% and most typically about 90-95%) with the corresponding sequences of endogenous or naturally occurring CHK, and possesses one or more of the functions of endogenous CHK. For example, a biologically active CHK fragment typically shares sequence homology with endogenous CHK protein in the domains important for biological activity, e.g., the tyrosine kinase domain, or SH2 domain. Biologically active fragments or analogs may be naturally occurring, recombinant, or synthetic, or may be derivatives

or fragments of peptides, e.g., those in which the N- or C-terminal group has been structurally modified.

CHK of the present invention is understood to specifically include CHK proteins having amino acid sequences analogous to the sequence of the endogenous CHK. Such proteins are defined herein as CHK analogs. An "analog" is defined herein to mean an amino acid sequence with sufficient identity to the amino acid sequence of endogenous CHK protein to possess the biological activity of the protein. For example, an analog of a polypeptide can be introduced with "silent" changes in the amino acid sequence wherein one or more amino acid residues differ from the amino acid sequence of CHK, yet possess kinase activity or associates with ErbB-2. Examples of such differences include additions, deletions or substitutions of residues. Also encompassed by the present invention are proteins that exhibit greater or lesser biological activity of CHK protein.

The present invention also encompasses biologically active fragments of CHK protein. Such fragments can include only a part of the full-length amino acid sequence of CHK yet possess biological activity. As used herein, a "biologically active fragment" means a fragment that can exert a biological or physical effect of the full-length protein, or has a biological characteristic, e.g., antigenicity, of the full-length protein. For example, a biologically active fragment of CHK can bind to ErbB-2 at, or near Tyr^{1253}, resulting in the inhibitation of neoplastic cell growth. Another example of biological activity, as defined herein, is the ability of a CHK fragment to specifically bind to antibody, (e.g., antigenicity). The antigenicity of a peptide fragment can be determined, for example, as described in Geysen, *et al.*, WO 84/03564, the teachings of which are herein incorporated by reference. Also encompassed by the present invention is the ability of CHK fragments to elect an immune response (e.g., imunogenicity). Such activities and characteristics are described above.

Specifically encompassed by the present invention are CHH peptides comprising the amino acid sequence of the SH2 domain of CHK which specifically binds at or to Tyr^{1253} of ErbB-2. Such fragments can be produced by amino and carboxyl terminal deletions as well as internal deletions. Also included are active fragments of the protein as obtained by enzymatic digestion. Such peptide fragments can be tested for biological activity as described herein.

"Derivatives" and "variants" of CHK are CHK proteins that have been modified. They include CHK proteins that have been modified by alterations in their amino acid sequence. They also include truncated and hybrid forms of CHK. "Truncated" forms are shorter versions of CHK, typically modified so as to remove the C-terminal regions which effect binding or secretion. "Hybrid" or "chimeric" forms are CHK proteins that are composed of one or more CHK proteins combined with one or more other proteins, such as another kinase.

Other biologically active derivatives or analogs of CHK proteins and peptides, referred to herein as peptide mimetics, can be designed and produced by techniques known to those of skill in the art. (See e.g., U.S. Patent Nos. 4,612,132; 5,643,873 and 5,654,276, the teachings of which are herein incorporated by reference). These CHK mimetics are based on the CHK amino acid sequence. These CHK peptide mimetics possess biological activity similar to the biological activity of the corresponding peptide compound, but can possess a "biological advantage" over the corresponding CHK protein or peptide with respect to one, or more, of the following properties: solubility, stability, and susceptibility to hydrolysis and proteolysis.

Methods for preparing peptide mimetics include modifying the N-terminal amino group, the C-terminal carboxyl group, and/or changing one or more of the amino linkages in the peptide to a non-amino linkage. Two or more such modifications can be coupled in one peptide mimetic inhibitor. Examples of modifications of peptides

to produce peptide mimetics are described in U.S. Patent Nos: 5,643,873 and 5,654,276, the teachings of which are incorporated herein by reference.

Variants can be produced using methods discussed below. The CHK gene can be mutated *in vitro* or *in vivo* using techniques well known to those of skill in the art, for example, site-specific mutagenesis and oligonucleotide mutagenesis. Manipulations of the CHK protein sequence can be made at the protein level as well. Any of numerous chemical modifications can be carried out by known techniques including, but not limited to, specific chemical cleavage by cyanogen bromide, trypsin and papain. It can also be structurally modified or denatured, for example, by heat or by being immobilized on a solid surface.

The amino acid sequences of the CHK proteins of the present invention can be altered to optimize CHK association with ErbB-2 by methods known in the art by introducing appropriate nucleotide changes into native or variant DNA encoding the CHK, or by *in vitro* synthesis of the desired CHK. Alterations can be created outside of or within the CHK SH2 domain.

In general, mutations can be amino acid substitutions, amino acid insertions or amino acid deletions, and can be conservative or non-conservative. The mutations can be at or near (e.g., within 5 or 10 amino acids of) the SH2 binding domain. More preferably, DNA encoding a CHK amino acid sequence variant is prepared by site-directed mutagenesis of DNA that encodes a variant or a nonvariant version of CHK. Site-directed (e.g., site-specific) mutagenesis allows the production of CHK variants through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed.

Typically, a primer of about 20 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both

sides of the junction of the sequence being altered (e.g., Figure 3, SEQ ID NO: 3). In general, the techniques of site-specific mutagenesis are well known in the art, as exemplified by publications such as Edelman *et al.*, *DNA*, 2:183 (1983). The site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing *et al.*, *Third Cleveland Symposium on Macromolecules and Recombinant DNA*, A. Walton, ed., Elsevier, Amsterdam (1981). This and other phage vectors are commercially available and their use is well-known to those skilled in the art. A versatile and efficient procedure for the construction of oligonucleotide directed site-specific mutations in DNA fragments using M13-derived vectors was published by Zoller, M.J. and Smith, M., in *Nucleic Acids Res.* 10:6487-6500 (1982). Also, plasmid vectors that contain a single-stranded phage origin of replication can be employed to obtain single-stranded DNA (Veira *et al.*, *Meth. Enzymol.*, 153:3 (1987)). Alternatively, nucleotide substitutions can be introduced by synthesizing the appropriate DNA fragment *in vitro*, and amplifying it by PCR procedures known in the art.

In general, site-specific mutagenesis can be performed by first obtaining a single-stranded vector that includes within its sequence a DNA sequence that encodes the relevant protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically, for example, by the method of Crea *et al.*, (*Proc. Natl. Acad. Sci. USA*, 75:5765 (1978)). This primer can then be annealed with the single-stranded protein sequence-containing vector, and subjected to DNA-polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex

vector can then be used to transform appropriate host cells such as JM101 cells, and clones can be selected that include recombinant vectors bearing the mutated sequence arrangement. Thereafter, the mutated region can be removed and placed in an appropriate expression vector for protein production.

The PCR technique can also be used in creating amino acid sequence variants of a CHK. When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers can be designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer is preferably identical to a stretch of sequence on the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 500 nucleotides from that of the first, such that in the end, the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

If the ratio of template to product material is extremely low, the vast majority of product DNA fragments will incorporate the desired mutation(s). This product can be used to replace the corresponding region in the plasmid that served as the PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments

simultaneously to the vector fragment in a three (or more) part ligation.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells *et al.*, (*Gene*, 34:315 (1985)). The starting material can be the plasmid (or vector) comprising the CHK DNA to be mutated. The codon(s) within the CHK to be mutated are identified. There must be unique restriction endonuclease sites on each side of the identified mutation site(s). If such restriction sites do not exist, they can be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the CHK DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated CHK DNA sequence, which can be expressed to produce CHK with altered binding activity.

Specifically encompassed by the present invention are reagents and methods for detecting and diagnosing breast cancer. Such reagents and methods work by detecting the presence or absence of CHK protein in mammalian cells wherein detection of the presence of (e.g., the expression of) CHK is indicative of breast cancer. A biological sample to be tested for the presence or absence of CHK protein is obtained from the mammal. Typically, the sample is breast tissue or tissue adjacent to the breast. The tissue sample can include lymph nodes. The sample is

typically obtained by biopsy techniques well known to those of skill in the art.

CHK protein expression can be detected in a tissue sample by immunohistochemical techniques as described herein. For example, the tissue can be imbedded in paraffin or frozen and sectioned into thin slices, typically mounted on microscope slides. The tissue is contacted with an anti-CHK antibody under conditions suitable for the anti-CHK antibody to specifically bind to CHK present in the tissue sample as described herein. The anti-CHK antibodies can be monoclonal or polyclonal. The antibody can be detectably labeled, for example, with a fluorescent dye. Alternatively, a second antibody that is detectably labeled can be used. For example, if the first antibody is a mouse anti-CHK antibody, a second antibody can be detectably labeled rabbit anti-mouse. Techniques for producing, purifying and labeling antibodies are well-known to those of skill in the art. For example, the peptide of Figure 3, (SEQ ID NO: 4) can be used to raise specific anti-CHK antibodies.

Expression of CHK protein can also be detected by Western blot (immunoblot) analysis using anti-CHK antibodies as described herein. Additionally, the expression of CHK protein can be detected by immunoprecipitation using anti-CHK antibodies, also as described herein. Additional techniques suitable for use to detect the presence of CHK protein includes e.g., immunofluorescence staining, confocal staining and ELISA when using soluble lysates. Such techniques are also well known to those of skill in the art.

Detection of the presence or absence of CHK can also be accomplished by the detection of the presence or absence of nucleic acids, either DNA or RNA, encoding the CHK protein in a biological sample. The biological sample, e.g., breast tissue, can be prepared in a manner that renders the nucleic acid encoding CHK available for hybridization with a nucleic acid probe that specifically hybridizes with a nucleic acid sequence that encodes all,

or a portion, of CHK. For example, Northern blot analysis, or Southern blot analysis can be used to detect the presence of CHK RNA or DNA in a biological sample. These techniques are well-known to those of skill in the art. See e.g., Sambrook, J. *et al.*, *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), or Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, J. Wiley and Sons, Inc./Wiley Interscience, New York, N.Y. (1992). For example, the standard Southern blot methods includes extracting genomic DNA from the sample, and digesting the genomic DNA with suitable restriction enzymes to obtain DNA fragments. The DNA fragments are then separated by electrophoretic means on e.g., agarose gels and transferred to nylon membranes which are exposed to detectably-labeled probes under conditions sufficient for the probes to specifically hybridize to nucleic acids encoding CHK. Detection can be accomplished by, e.g., autoradiography, spectrometry or fluorometry.

Nucleic acid probes useful in the present invention comprise at least about 15 nucleotides, typically about 21 to 45 nucleotides and most typically about 100 nucleotides. This number of nucleotides typically provides the minimal length required of a probe that would specifically hybridize to a CHK-encoding sequence. The probes are of a specificity and sufficient length to form stable hybrid duplexes with the target sequence under stringent conditions. An example of a CHK probe is SEQ ID NO: 3. As used herein, "stringent conditions" are defined as conditions under which specific hybrid duplexes will be stable and maintained and under which non-specific hybrid duplexes will be not be stable (e.g., specific hybrid duplexes will be stable during wash conditions while non-specific hybrid duplexes will be eluted during wash conditions. Probes and conditions useful in the present invention are described in WO 93/15201, entitled "Novel Protein Tyrosine Kinases", the teachings of which are herein incorporated in their entirety by reference (also

see Fig. 3, SEQ ID NO: 3, which is a nucleic acid sequence encoding a CHK peptide). Techniques for identifying probes and conditions of stringency (e.g., moderate or high) are also well-known to those of skill in the art and e.g., are described in Sambrook, J. *et al.*, *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), or Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, J. Wiley and Sons, Inc./Wiley Interscience, New York, N.Y. (1992).

It is important to note that the nucleotide sequences of probes that are useful in the present invention need not be fully complementary to the target sequence. Probes need only be substantially complementary. As defined herein, "substantially complementary" means that the probe sequence is sufficiently similar in sequence identity to the target sequence that the probe specifically hybridizes with the target sequence under specified conditions. For example, non-complementary bases can be interspersed within the probe sequence, or the probe can be longer or shorter than the target sequence, provided that the probe still specifically hybridizes with the target sequence.

Detection of hybrid duplexes is typically accomplished by the use of detectably labeled probes. Such labels and methods of labeling probes are well-known to those of skill in the art. For example, labels can be radiolabels, chemiluminescent labels, fluorescent labels, biotin, enzymes or other labels known to those of skill in the art. Alternatively, the probe can be unlabeled but detectable by subsequent binding or hybridization to a second, detectably labeled molecule.

Detection of nucleic acids encoding CHK can also be accomplished by amplification techniques which directly amplify the target nucleic acid present in a sample, for example, by polymerase chain reaction (PCR) (see e.g., Saiki, *et al.*, *Science*, 230:1350-1353 (1986)) or ligase chain reaction (LCR) (ee e.g., Weiss, R., *Science*, 254:1292-1293 (1991)). Such amplification techniques can

also be used as preliminary steps for detection techniques described above.

In situ hybridization analysis of tissue samples can also be used to detect the presence or absence of CHK in a biological sample. Such techniques are also well-known to those of skill in the art. (See for example, Sure Site II System™ hybridization kit by NoVagen.)

The present invention also encompasses the use of the CHK proteins and nucleic acids encoding these proteins as a basis of rational drug design to produce biologically active CHK analogs that have substantially comparable, or lesser or greater biological activity of CHK. Also encompassed are the use of the CHK proteins to identify small molecules which interact with CHK and thus, can act as agonists, antagonists or inhibitors of CHK activity.

A further embodiment encompassed by the present invention includes methods of inhibiting neoplastic (tumor) cell growth by supplying CHK to cells. Specifically encompassed by the present invention are therapeutic methods that inhibit ErbB-2 mediated-breast cancer cell growth and can therefore be used for prophylaxis and treatment of breast cancer. Cells that are in need of CHK and are supplied with, or receive CHK protein, are referred to herein as target or recipient cells. The recipient cells are either substantially deficient in CHK (e.g., fail to produce an amount of CHK sufficient to suppress neoplastic growth, or hyperplasia, which is abnormal growth) or produce adequate amounts of CHK, but the CHK produced is functionally abnormal (e.g., the CHK lacks biological activity to suppress neoplastic growth). As defined herein, the term "inhibit" means either to completely suppress or prevent (e.g., prophylactically prevent) neoplastic cell growth or to substantially or significantly decrease neoplastic or hyperplastic cell growth. Inhibition or decrease of cancer cell growth or hyperplasia can be measured as described herein, e.g., by comparing growth of breast tumor cells that have been supplied with CHK to growth of breast tumor cells that have

not been supplied with CHK, and by other methods well-known to those of skill in the art.

CHK protein, peptide or a biologically active fragment thereof, or a CHK analog or derivative, can be supplied to mammalian breast tissue that manifests neoplastic cell growth, or is at risk of producing neoplastic cell growth e.g., hyperplastic tissue. CHK can be supplied to (e.g., introduced into) the target recipient cells by methods well-known to those of skill in the art. For example, CHK can be introduced into recipient cells by injection of a pharmaceutical composition that contains an effective amount of CHK in a physiologically compatible solution, or by a liposome preparation that contains an effective amount of CHK. Specifically encompassed by this invention is the topical application of liposomes in a cream or ointment which contain an effective amount of CHK. An effective amount of CHK is defined herein as an amount of CHK which inhibits neoplastic or hyperplastic cell growth, specifically ErbB-2 mediated breast cancer cell growth.

Suitable physiologically acceptable carriers include but are not limited to water, salt solutions, alcohols, gum arabic, vegetable oil, benzyl alcohols, polyethylene glycols, gelatine, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers and the like which do not deleteriously react with the active compounds. They can also be combined, where desired, with other active agents, e.g., enzyme inhibitors, to further reduce metabolic degradation.

For topical application, the preparations can be employed as nonsprayable forms, viscous semi-solid or solid forms comprising a carrier compatible with topical application and having a dynamic viscosity preferably

greater than water. Suitable formulations include but are not limited to solutions, suspensions, emulsions, creams, foams, ointments, powders, liniments, salves, aerosols, etc., which are, if desired, sterilized or mixed with auxiliary agents, e.g., preservatives, stabilizers, wetting agents, buffers or salts for influencing osmotic pressure, etc. For topical application, also suitable are sprayable aerosol preparations wherein the active ingredient, preferably in combination with a solid or liquid inert carrier material, is packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant, e.g., pressurized air or some other propellant well-known in the art. Also included are transdermal patches as discussed, for example, in WO 93/07870, the teachings of which are incorporated by reference.

Alternatively, CHK can be supplied to the recipient cells by introducing a nucleic acid sequence encoding CHK, or a biologically active fragment, analog, or derivative of CHK which is then expressed in the recipient cells. Methods of introducing nucleic acids encoding specific proteins such as CHK are well-known to those of skill in the art. For example, expression vectors can be designed and produced that contain a nucleic acid insert which encodes CHK or a biologically active fragment of CHK. Methods to construct these expression vectors are well-known to those of skill in the art. For example, described herein is an expression vector comprising vaccinia virus useful for expressing a DNA insert encoding CHK. In addition to vaccinia virus, other virus or plasmid vectors, such as retroviruses or plasmid vectors, can be used to introduce nucleic acids encoding CHK into recipient cells. Additionally, naked DNA can be injected into recipient cells, or methods such as elctroporation, co-precipitation or a "gene gun" can be used to deliver the DNA to the recipient cells.

Other techniques using naked plasmids or DNA, and cloned genes encapsidated in target liposomes or in erythrocyte ghosts, can be used to introduce the receptor

into the host (Friedman, T., *Science*, 244:1275-1281 (1990); Rabinovich, N.R. *et al.*, *Science*, 265:1401-1404 (1994)). The construction of expression vectors and the transfer of vectors and nucleic acids into various host cells can be accomplished using by using commercially available kits, or genetic engineering techniques well known in the art, such as those described in Sambrook, J. *et al.*, *Molecular Cloning*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) or Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, J. Wiley and Sons, Inc./Wiley Interscience, New York, N.Y. (1992) the teachings of which are hereby incorporated, in their entirety, by reference.

Cells from a patient's tumor can be analyzed by the diagnostic methods described above to determine the presence of CHK or to determine the biological activity of CHK present in their cells. A vector as described herein, containing a nucleic acid encoding CHK and operably linked to expression control elements required for the expression of a protein in the recipient cells, is introduced into the patient, either at the site of the tumor, or by intravenous or other parenteral injection, so as to reach any tumor cells that may have metastasized to other sites. The introduction may be repeated as necessary in order to achieve the desired effect of inhibiting neoplastic growth. A description of techniques that may be used to specifically target breast cells is described in EP 0 699 754 A1, the teachings of which are herein incorporated by reference.

Thus, as a result of the work described herein, novel methods of detecting and inhibiting breast cancer, or hyperplastic growth that may result in cancer, are now available.

The following examples more specifically illustrate the invention and are not intended to be limiting in any way.

EXAMPLES

EXAMPLE 1: EXPRESSION OF CHK IN HUMAN BREAST CANCER TISSUE

Materials: Recombinant heregulin (rHRG-1, 177-244), rabbit polyclonal anti-ErbB-2 antibodies, and 3E8-monoclonal anti-ErbB-2 antibodies, were obtained from Genentech, Inc. (San Francisco, CA) (Levi, A.D. *et al.*, *J. Neurosci.*, 15:1329-1340 (1995)). EGF and IL-6 were purchased from Collaborative Biomedical Products (Bedford, MA) and from R & D Systems (Minneapolis, MN) respectively. Monoclonal anti-phosphotyrosine antibody (PY20) conjugated to horse radish peroxidase (HRP) was obtained from Zymed, Inc. (San Francisco, CA). Polyclonal antibodies for EGF-R, ErbB-3, ErbB-4 and polyclonal anti-CHK (anti-LSK) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GST monoclonal antibodies were purchased from Pharmacia Biotech, Inc. (Piscataway, NJ). GST fusion proteins containing the NH2-SH2 domain of p85 PI3 kinase and SH2-SH2-SH3 domains of PLC-1 were obtained from Santa Cruz Biotechnology. The primers for the polymerase chain reaction (PCR) were synthesized by an automated DNA Synthesizer (Applied Biosystems, Model 394). Reagents for electrophoresis were obtained from BioRad (Hercules, CA). ECL reagents were purchased from Amersham Corp. (Arlington Heights, IL). All other reagents were purchased from Sigma (St. Louis, MO).

EXPERIMENTAL PROCEDURES: Immunohistochemical staining was performed on paraffin-embedded 5 mm-thick tissue sections of human breast cancer. Sections were deparaffinized in xylene and then incubated in decreasing concentrations of ethyl alcohol. After several rinses in water, the slides were incubated in methanol/hydrogen peroxide (1:4), briefly rinsed in water and then in PBS (pH 7.6). Subsequent immunohistochemical staining was performed using a 1:100 dilution in PBS of rabbit anti-CHK antisera (1 hour incubation) followed by the addition of the secondary antibodies, peroxidase-conjugated rabbit anti-mouse IgG (Sigma, St. Louis, MO) at 50 μg/ml in PBS.

Analyses of CHK expression in human breast cancer tissues at different stages were performed using immunohistochemistry on paraffin sections. Results (Table 1) revealed that CHK is expressed in the majority of breast cancers, but was not detected in normal adjacent tissue.

TABLE 1

CHK Expression in Primary Breast Cancer Tissues	
BREAST CANCER PATIENTS	NO. PATIENTS (+) FOR CHK
Stage I	32/41
Stage II	34/35
Stage III	4/4
Normal Breast, Fibroadenoma	0/19
Immunohistochemical staining was performed on paraffin embedded sections of infiltrating ductal carcinoma using anti-CHK antibodies.	

EXAMPLE 2: CHK IS ASSOCIATED WITH ACTIVATED ErbB-2 UPON STIMULATION WITH HRG

Experiments were performed using the T47D breast cancer cell line and the GST-fusion protein containing the SH2 domain of CHK (CHK-SH2). T47D cells express the ErbB family receptors and the CHK protein as observed by immunohistochemistry. The T47D human breast cancer cell line was obtained from ATCC (American Type Culture Collection, Rockville, MD). T47D cells were grown in RPMI-1640 medium (GIBCO/BRL, Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and 3.5 μg/ml insulin (Sigma). Prior to stimulation with HRG, cells were starved overnight in media containing 1% FBS and then for 4 hours in serum-free medium. The starved cells were then stimulated with HRG (10 nM) for the indicated time points (Fig. 1A to Fig. 1D). Cells were lysed, and the supernatants were incubated with the purified CHK-SH2 fusion protein (Figs. 1A, 1B) or with the 3E8 monoclonal antibody to ErbB-2 (Figs. 1C, 1D). The co-precipitated

proteins were analyzed on 7% SDS-PAGE, and immunoblotted with PY20 (Figs. 1A, 1C).

As shown in Fig. 1A, a tyrosine-phosphorylated 185 kDa protein was associated with CHK-SH2 within 2 minutes of the HRG stimulation. The association of the 185 kDa with CHK-SH2 was maximal at 2-8 minutes after HRG stimulation and then gradually decreased. In order to determine whether the 185 kDa protein was ErbB-2, the blot was deprobed and reblotted with polyclonal anti-ErbB-2 antibody. As shown in Fig. 1B, the 185 kDa protein was confirmed to be the ErbB-2 protein. These results indicated that the CHK protein can interact with the HRG-activated ErbB-2 receptor.

When lysates from HRG-treated cells were immunoprecipitated with the 3E8 monoclonal anti-ErbB-2 antibody, the pattern of the phosphorylated ErbB-2 was different from that of the ErbB-2 precipitated with the SH2 domain of CHK (compare Fig. 1C with Fig. 1A). Blotting of the same samples with the polyclonal anti-ErbB-2 antibody (Fig. 1D) confirmed these observations.

CHK-SH2 fusion proteins also precipitated other, as yet unidentified, tyrosine-phosphorylated proteins as shown in Fig. 1A. However, these phosphorylated proteins were also precipitated from the unstimulated cells and their phosphorylation pattern did not appear to change over the time course of these studies.

EXAMPLE 3: THE ASSOCIATION OF CHK WITH ErbB-2 IS SPECIFIC FOR HRG STIMULATION

In order to determine whether the observed association of CHK with ErbB-2 was receptor-specific and stimulus-specific, experiments were performed to analyze whether CHK could associate with either the EGF-R or IL-6 receptors which are both known to be expressed in T47D cells. The association of CHK-SH2 with ErbB-2 in lysates from HRG, EGF and IL-6 stimulated cells was compared. T47D cells were serum starved as described above and then activated either with HRG (10 nM) for 8 minutes or with EGF

(100 ng/ml) or IL-6 (100 ng/ml) for 5 min. The experimental time points and the concentrations of EGF and IL-6 were optimized in initial kinetic studies. The stimulated cells were lysed and precipitated with the CHK-SH2 fusion protein as described above. The precipitates were then analyzed on SDS-PAGE and immunoblotted with PY20 antibodies or with polyclonal anti-ErbB-2 antibodies. Only HRG stimulation induced the association of ErbB-2 with the purified CHK-SH2 fusion protein. EGF or IL-6 stimulation failed to induce CHK-SH2 association either to ErbB-2, or to the EGF-receptor or the IL-6 receptor.

The association of ErbB-2 with other SH2 domain-containing signaling molecules such as p85 of PI3-kinase, PLC-1 or Shc was also examined. The SH2-SH2-SH3 domain of PLC-1 was found to be associated with the HRG-activated ErbB-2 as well as with SH2. The SH2 domain of PI3-kinase precipitated ErbB-2, probably as a result of the ErbB-2 heterodimerization with ErbB-3. Taken together, these results indicate that ErbB-2 associates with all three signaling molecules in HRG-activated T47D cells.

Experiments were also performed to show that the SH3 domain of CHK is not involved in the interaction between CHK and ErbB-2.

The potential involvement of other domains of CHK in the interaction with ErbB-2 was also examined. GST-fusion proteins containing the SH3 domain of CHK (CHK-SH3), the N-terminal domain plus SH3 domain (NH2 -SH3), the SH3 and SH2 domains of CHK (SH3-SH2), the SH2 domain of CHK as well as the GST protein alone were prepared as follows.

GENERATION OF FLAG-CHK CONSTRUCT IN PCDNA3 VECTOR: The CHK cDNA (1.6 kb, SEQ ID NO: 1) was cloned into *Eco*RI sites in the pCDNA3 neo vector. The nucleotide sequence for the Flag epitope (Asp-Tyr-Lys-Asp-Asp-Asp-Asp- Lys, SEQ ID NO: 5) was added to the 5' end of the ORF (open reading frame) of the CHK cDNA sequence by PCR, using 1.6 kb CHK cDNA as a

template. The 5' sense primer included a *Bam*HI restriction site, ATG initiation codon, the Flag sequence, and CHK sequence from nucleotides #269 to #295 of SEQ ID NO: 1, Fig. 2 (Bennett, B.D. *et al.*, *J. Biol. Chem.*, 269:1068-1074 (1994)), the teachings of which are herein incorporated, in their entirety, by reference). The 3'-antisense primer was composed of CHK sequences from nucleotides #510 to #481 of SEQ ID NO: 1. The PCR product was double digested with *Bam*HI and *Bst*EII (New England Bio Labs, Beverly, MA), gel-purified and then cloned into *Bam*HI and *Bst*EII sites in the pDNA3 neo-Flag-CHK. The construct was analyzed by restriction mapping and nucleotide sequencing.

TRANSFECTION: Transfection of MCF-7 cells was performed using the Lipofectamine™ (Gibco/BRL, Bethesda, MD) according to the manufacturer's protocol. The transfected cells were selected in 1.2 mg/ml G418 (Sigma, St. Louis, MO). Positive transfectants were chosen based on their immunoreactivity on Western blots probed with polyclonal anti-CHK and monoclonal anti-Flag (M5) antibodies (Eastman Kodak Company, New Haven, CT).

CONSTRUCTION AND PURIFICATION OF GST-FUSION PROTEINS OF CHK: To express the NH2-SH3 and SH3-SH2 domains of CHK as GST-fusion proteins, the corresponding DNA sequences were amplified by PCR with sense and antisense primers of CHK cDNA which contained BamHI and EcoRI restriction sites. For the NH2-SH3 construct, we used the sense primer from nucleotides #4 to #27 SEQ ID NO: 1 and the antisense primer from nucleotides #343 to #321 of SEQ ID NO: 1. For the SH3-SH2 construct, the sequence from nucleotides #127 to #150 of SEQ ID NO: 1 was used as the sense primer and nucleotides #657 to #634 of SEQ ID NO: 1 served as the antisense primer. The DNA fragments obtained from PCR were restriction digested with *Bam*HI and *Eco*RI and ligated into the pGEX-2T vector (Pharmacia, Uppsala, Sweden). The sequence and orientation were confirmed by sequencing both strands. Construction of the GST-fusion proteins of

CHK-SH2 and CHK-SH3 were performed as described in Jhun, B.H. *et al.*, *J. Biol. Chem.* 270:9661-9666 (1995).

GST-fusion proteins were produced by the induction of transformed bacteria using 10 mM isopropylthiogalactoside (IPTG), and purified on a large scale by affinity chromatography on glutathione-sepharose beads (Pharmacia, Uppsala, Sweden) according to the manufacturer's protocol.

HRG-stimulated T47D cell lysates were incubated with the different GST-fusion proteins, analyzed by SDS-PAGE, and immunoblotted either with PY20, rabbit anti-ErbB-2 antibody or with anti-GST antibody. Neither the SH3 domain of the CHK protein nor the NH2-SH3 domain precipitated ErbB-2. Binding to ErbB-2 was detected only in the presence of the CHK-SH2 and CHK-SH3-SH2 fusion proteins. As expected, no binding was detected when the same lysates were incubated with the GST protein alone. The amounts of the different fusion proteins loaded on the gel were comparable. These results confirm that CHK can interact with the HRG-stimulated ErbB-2 in a specific manner via its SH2 domain.

EXAMPLE 4: IN VIVO ASSOCIATION OF INTACT CHK WITH ErbB-2

The MCF-7 human breast cancer cell line was obtained from ATCC (American Type Culture Collection, Rockville, MD). The MCF-7 cells were grown in MEM (GIBCO, Bethesda, MD) supplemented with 10% FBS, 5 μg/ml insulin (Sigma, St. Louis, MO), 1 mM non-essential amino acids and 1 mM sodium pyruvate. Prior to stimulation, cells were starved overnight in media containing 1% FBS and then for 4 hours in serum-free medium.

To further confirm the association of ErbB-2 with CHK, the CHK protein was overexpressed in MCF-7 breast cancer cells. CHK expression in MCF-7 cells was detected only by PCR analysis. Expression of the ErbB receptor family in MCF-7 cells was similar to that observed in T47D cells. Stable transfections were performed using the Flag-CHK pCDNA3 *neo* construct as described above. The transfected cells were analyzed for CHK expression by Western blot

using anti-Flag and anti-CHK antibodies and also by immunofluorescence using confocal microscopy. MCF-7 cells transfected with Flag-CHK pCDNA3 *neo* (Flag-CHK), MCF-7 cells transfected with the pCDNA3 *neo* vector alone, or untransfected MCF-7 control cells, were stimulated with HRG and then lysed.

Immunoprecipitation studies were performed as follows. Approximately 5 x 10^6 cells/plate were starved overnight in media containing 1% FBS, followed by additional starvation in serum-free medium for 4 hours at 37°C. The starved cells were then stimulated with 10 nM HRG for 8 minutes or with 100 ng/ml EGF or 100 ng/ml IL-6 for 5 minutes at room temperature. The stimulation was terminated by the addition of an ice-cold lysis buffer (0.1% SDS, 1% Triton X-100, in Tris-buffered saline containing 10% glycerol, 1 mM EDTA, 0.5 mM Na_3VO_4 (sodium orthovanadate), 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 10 mM leupeptin). Lysates were pre-cleared by centrifugation (14,000 rpm, 15 min) and then incubated for 90 minutes at 4°C with 10 μg of GST-fusion proteins coupled to glutathione-sepharose beads. The beads were washed three times with the lysis buffer. For the immunoprecipitation experiments, polyclonal anti-CHK antibody, monoclonal anti-ErbB-2 antibody, 3E8 (10 μg/ml), polyclonal anti-ErbB-3 antibody (10 μg/ml) or polyclonal anti-ErbB-4 antibody (10 μg/ml) were used. SDS-sample buffer was added to the samples and analyzed on 7% polyacrylamide SDS-PAGE. Proteins were transferred onto nitrocellulose or Immobilon-PTM (Millipore, Inc., Bedford, MA) membranes. Bound proteins were immunoblotted with anti-phosphotyrosine antibody (PY20), polyclonal anti-ErbB-2 antibody, or polyclonal anti-CHK, EGF-R, ErbB-3 or ErbB-4 antibodies. The blots were developed using the enhanced chemiluminescence (ECL) system (Amersham). Blots were stripped for 30 minutes at 55°C in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7), according to the manufacturer's protocol (Amersham, Arlington Heights, IL).

The 185 kDa tyrosine-phosphorylated protein was immunoprecipitated with anti-Flag antibodies or anti-CHK antibodies only in HRG-stimulated Flag-CHK transfected cell lysates, but not in the untransfected MCF-7 cell lysates or the MCF-7 cell lysates transfected with the pcDNA3-neo Flag vector alone. Blotting with the anti-ErbB-2 antibody confirmed that the co-precipitated 185 KDa protein was indeed the ErbB-2. Analysis of the total lysates from the same experiment revealed that the ErbB-2 was tyrosine-phosphorylated as a result of the HRG stimulation in the Flag-CHK cells as well as in the MCF-7 untransfected cells. The expression of ErbB-2 appeared to be equal in both the Flag-CHK and MCF-7 cells. Taken together, these in vitro and in vivo data indicate that the HRG-stimulated ErbB-2 associates with CHK through the SH2 domain.

EXAMPLE 5: INVOLVEMENT OF OTHER ErbB-2 FAMILY MEMBERS IN THE INTERACTION WITH CHK

To further investigate the possible involvement of other members of the ErbB family in the observed interaction between CHK and ErbB-2, co-immunoprecipitation experiments using MCF-7 cells transfected with Flag-CHK were performed. Flag-CHK transfected cells were stimulated with HRG, and then lysed and immunoprecipitated with anti-CHK antibody as described above. The immunocomplexes were separated by SDS-PAGE and immunoblotted with anti-ErbB-2 antibody or with anti-ErbB-3 antibody. The results indicated that anti-CHK antibody immunoprecipitated the HRG-activated ErbB-2. In contrast, no detectable ErbB-3 was found. However, the possibility that very low amounts of ErbB-3 were present in the precipitates as a result of the heterodimerization with the ErbB-2 receptor upon HRG stimulation cannot be excluded.

It was also investigated whether ErbB-4 interacted with CHK under these conditions, however, findings indicated that ErbB-4 was not involved in the ErbB-2-CHK association.

In order to confirm the presence and phosphorylation of the ErbB-3 as well as the heterodimerization of ErbB-3 with ErbB-2 in the Flag-CHK transfected cells, lysates from HRG-stimulated Flag-CHK cells were immunoprecipitated with anti-ErbB-3 antibodies or with anti-ErbB-2 antibodies. Both ErbB-3 and ErbB-2 were tyrosine-phosphorylated upon HRG stimulation and the formation of ErbB-2-ErbB-3 heterodimers was demonstrated by the presence of ErbB-2 in the precipitates of the anti-ErbB-3 antibodies. However, under these conditions, ErbB-3 was not detected in the samples immunoprecipitated with anti-ErbB-2 antibody. Taken together, these observations indicate that upon HRG stimulation, heterodimerization of ErbB-3 with ErbB-2 receptors occurred in the transfected cells, suggesting that the ErbB signaling in these cells is not altered.

To determine whether EGF-R (ErbB-1) might be involved in ErbB-2-CHK interactions, Flag-CHK MCF-7 transfected cells were serum-starved and then stimulated with HRG (10 nM) or with EGF (100 ng/ml). The lysates were immunoprecipitated with anti-CHK antibodies and analyzed by SDS-PAGE. Only the tyrosine-phosphorylated ErbB-2 protein was immunoprecipitated with anti-CHK-antibodies in the HRG-stimulated lysates. No tyrosine-phosphorylated proteins were detected in the immunoprecipitates with anti-CHK antibodies from the EGF-stimulated cells. Reprobing of this blot with anti-ErbB-2 or with anti-EGF-R antibodies confirmed that neither of these receptors were present in the CHK immunoprecipitates.

As a control, immunoprecipitations with anti-EGF-R antibodies of the EGF-stimulated Flag-CHK cell lysates as well as of lysates from untransfected MCF-7 cells were performed. The EGF-R and the ErbB-2 proteins were present in the immunoprecipitates from the EGF-stimulated cells as a result of the EGF-ErbB-2 heterodimerization. Probing of the same blot with anti-ErbB-2 or anti-EGF-R antibodies confirm this observation.

These analyses indicate that CHK associates via its SH2 domain with HRG-stimulated ErbB-2. This association is

specific to HRG-stimulated ErbB-2 and does not appear to prominently involve other ErbB family members.

EXAMPLE 6: FUNCTIONAL ASSOCIATION OF CHK TO THE *NEC* (Val^{664}) AND *TEC* (Glu^{664}) EGF-ErbB-2 HYBRID RECEPTORS

ErbB-2 functions as a co-receptor for growth-regulatory molecules, including neuregulins. Replacement of the extracellular domain of ErbB-2 by the ligand binding domain of the receptor for EGF allows heterologous stimulation of the ErbB-2, which has been successfully exploited in signal transduction studies (Ben-Levy, R. *et al.*, *EMBO J.*, 13:3302-3311 (1994)). The transforming protein of ErbB-2, which contains a glutamine residue (Glu^{664}) instead of a valine (Val^{664}) residue, is a constitutively active receptor permanently coupled to signaling pathways. To confirm that the association of CHK with ErbB-2 is mediated by the intracellular domain of ErbB-2 and not by other members of the ErbB-2 family, chimeric proteins that include the extracellular domain of the EGF receptor and the transmembrane and cytoplasmic domains of the ErbB-2, termed NEC (Val^{664}), or the point-mutated cytoplasmic domain of ErbB-2 (Glu^{664}) termed TEC, (kindly obtained from Dr. Y. Yarden (Department of Chemical Immunology, the Weizmann Institute of Science, Rehovot, Israel); Peles, E. *et al.*, *J. Biol. Chem.*, 267:12266-12274 (1992)) were used in this study. It is important to note that ErbB-2 does not directly bind to any of the EGF-like ligands. However, EGF and HRG induce the tyrosine phosphorylation of ErbB-2, presumably by ligand-driven heterodimerization and transphosphorylation. NIH3T3 cells were stably transfected with the chimeric plasmid EGF-TEC-ErbB-2 or with the chimeric plasmid EGF-NEC-ErbB-2. TEC and NEC cells (4×10^6 cells/plate) were serum-starved and then left unstimulated, or stimulated with 100 ng EGF at room temperature for 5 minutes. The lysates were divided into two parts: one-half of the lysates were precipitated with the CHK-SH2 GST-fusion protein (10μg) for 90 minutes at 4°C. After washing, the precipitates were separated by

7% SDS-PAGE and immunoblotted with monoclonal anti-phosphotyrosine antibody (PY20), or with polyclonal anti-EGF-R antibodies. The other half of the lysates was immunoprecipitated using monoclonal antibodies for EGF-R for 16 hours at 4°C. The washed precipitates were run on 7% SDS-PAGE and blotted with PY20 or with anti-EGF-R antibodies.

CHK association with both EGF-stimulated and unstimulated NEC and TEC was analyzed. Upon EGF stimulation, CHK was found to associate via its SH2 domain with NEC, while its association with TEC was constitutive and not dependent on EGF stimulation. These results indicate that the CHK-SH2 domain specifically associates with the intracellular domain of ErbB-2.

EXAMPLE 7: INHIBITION OF THE CHK-SH2-ErbB-2 (also ErbB-2/neu) INTERACTION BY TYROSINE-PHOSPHORYLATED PEPTIDES

To identify the binding site of CHK-SH2 within the ErbB-2/neu receptor, a series of tyrosine-phosphorylated peptides were synthesized, derived from the five autophosphorylated tyrosine residue sites of the cytoplasmic domain of the ErbB-2/neu receptor (Tyr^{1028}, Tyr^{1144}, $Tyr^{1286/7}$ and Tyr^{1253}). These four tyrosine phosphorylated peptides: P_1 (ENPEY*LGLDV; SEQ ID NO:6), $P_{2,3}$ (DNLY*Y*WDQNS; SEQ ID NO:7), P_4 (QPEY*VNQSE; SEQ ID NO:8), and P_5 (AEEY*LVPQQ; SEQ ID NO:9) (Y*=Phosphotyrosine) were used to inhibit the interaction between the CHK-SH2 domain and the activated ErbB-2/neu receptor. COS cells were transiently transfected (using Lipofectamine™ reagents (GIBCO/BRL, Bethesda, MD), according to the manufacturer's instructions) with the transformed ErbB-2/neu plasmid that codes for the constitutively phosphorylated receptor (neu*). This protein differs only in one amino acid: it contains a glutamic acid at the transmembrane domain (residue 664) instead of a valine (Val^{664} -> Glu^{664}) (Ben-Levy, R. *et al.*, *EMBO J.*, 13:3302-3311 (1994); Ben-Levy, R. *et al.*, *J. Biol. Chem.*, 267:17304-17313 (1992)). Complexes of ErbB-2/neu and CHK-SH2 were indicated by the presence of

ErbB-2/neu in the washed CHK-SH2 GST-fusion protein precipitates. Of the four peptides, peptide P_1 (SEQ ID NO:6) most significantly inhibited complex formation. Inhibition by peptide P_5 (SEQ ID NO:9) was also found. To compare the relative abilities of the P_1 and P_5 peptides to inhibit the CHK-SH2-ErbB-2/neu interaction, various concentrations of peptides from 5-100 μM were tested. The results indicate that inhibition by the P_1 peptide was found to be much more significant throughout all the tested concentrations as compared to the other peptides, suggesting that binding of CHK is primarily at the P_1 site of the ErbB-2 receptor. Moreover, inhibition by the P_1 peptide was phosphorylation dependent, since the P_1 non-phosphorylated peptide had no inhibitory effect.

To further test the binding of CHK to the phosphorylated P_1 site, either the tyrosine phosphorylated P_1 peptide or the non-phosphorylated P_1 peptide was linked to Affi-Gel 15 beads, and the association of either CHK-SH2 GST-fusion protein or native CHK to the beads was analyzed. Peptide beads (15 μl bead volume) were incubated with 10 μg of CHK-SH2 for 1.5 hours at 4°C. The washed samples were separated on 10% SDS-PAGE. CHK-SH2 GST was associated in a phosphotyrosine-dependent manner to the phosphorylated P_1 peptide. Similar specificity was observed when the association of native CHK to the peptide beads was tested. 15 μl of beads were incubated with 1.5 ml of MCF-7, CHK-Flag-MCF-7 or neo-1 cell extracts for 1.5 hours at 4°C. After three washes, the precipitates were subjected to SDS-PAGE and Western blotting with anti-CHK antibodies. The phosphorylated P_1 peptide was able to associate with native CHK from extracts of CHK-Flag-MCF-7 transfected cells. No binding was observed in the MCF-7 or neo-1 lysates, which do not express CHK. Similar results were found when using CHK obtained from the vaccinia expression system. This specificity was in agreement with the peptide inhibition experiments, indicating a direct association between ErbB-2 and CHK-SH2 mediated by the P_1 tyrosine-phosphorylated site.

To confirm the significance of the phosphorylated peptide studies, the CHK-SH2 GST-fusion protein was tested to see if it could bind to the ErbB-2 receptor bearing only the P_1 phosphorylated site (Tyr^{1253}). Two constructs of the activated ErbB-2 receptor (neu*) were used to transfect the COS cells: (1) the P_1 construct which contains the extracellular and transmembrane domains of ErbB-2/neu and the P_1 binding site, and (2) the Y1253F construct which contains the full sequence of the constitutively activated ErbB-2, including a point mutation at the P_1 site (Y1253 -> Phe^{1253}). Both ErbB-2 constructs were tyrosine phosphorylated. Cell extracts from the same experiment were incubated with the CHK-SH2 GST-fusion protein, separated by SDS-PAGE and analyzed by Western blotting using ErbB-2 antibody. The CHK-SH2 GST precipitated the ErbB-2 in the COS cells transfected with the P_1 construct), while no association was found with the ErbB-2 carrying the point mutation on the P_1 site (YI253F). Therefore, the CHK-SH2 bound exclusively to the P_1-Tyr^{1253} site of ErbB-2.

To examine the *in vivo* association of the CHK molecule with the P_1-Tyr^{1253} site, the T7 polymerase vaccinia system was used to overexpress CHK. COS cells were first transiently transfected with the P_1 plasmid, and two days post-transfection, the cells were co-infected with the T7 polymerase virus alone, or with the T7 polymerase and CHK recombinant viruses. CHK was expressed in cells infected with the CHK recombinant virus, but not in cells that were infected with the T7 polymerase virus alone. Lysates from the same experiment were tested for the expression of the P_1-ErbB-2 molecule using anti-ErbB-2 antibody.

To demonstrate the *in vivo* association between P_1-ErbB-2/neu and CHK, the same cell extracts were immunoprecipitated using CHK antibody and then immunoblotted with ErbB2/neu antibody. P_1-ErbB-2/neu was present only in precipitates from the CHK-expressing cells and not in those of the T7-infected cells. Taken together,

these results indicate an *in vivo* association between CHK and the P_1 binding site (Tyr^{1253}) of ErbB-2.

EXAMPLE 8: GENERATION AND CHARACTERIZATION OF MCF-7 CELLS STABLY TRANSFECTED WITH CHK cDNA

In order to study the biological function(s) of CHK in human mammary epithelial cells, two known breast cancer cell lines, MCF-7 and T47D, were chosen. Both cell lines, obtained from American Type Culture Collection (ATCC, Rockville, MD), are well established in the field of breast cancer research and used extensively as models (Gras-Porta, D. *et al.*, *Mol. Cell. Biol.*, 15:1182-1191 (1995); Azijsen, R.M. *et al.*, *Mol. & Cell Biol.*, 16:2554-2560 (1996)). The expression of CHK in both these cell lines was analyzed. While T47D cells expressed CHK mRNA and protein as detected by Northern and Western blot analyses respectively, CHK expression in MCF-7 cells was detected only by PCR without evidence for significant levels of protein using immunoprecipitation or Western blotting. MCF-7 cells stably transfected with CHK cDNA that expressed CHK mRNA and CHK protein were generated. The MCF10-A cell line was used as a model for normal breast epithelial cells (Soule, H.D. *et al.*, *Cancer Research*, 50:6075-6086 (1990). These cells lacked expression of CHK, as evaluated by Northern blot, PCR and Western blot.

Stable transfections of MCF-7 cells were performed using the FLAG-CHK-pcDNA3neo construct or the pcDNA3neo vector as a control. CHK protein can be detected either by CHK specific antibodies or FLAG monoclonal antibodies. The proliferation rate of MCF-7 cells transfected with the FLAG-CHK-pcDNA3neo construct overexpressing CHK protein was significantly reduced ($p<0.001$) compared to the untreated MCF-7 cells or to the MCF-7 cells transfected with the FLAG pcDNA3neo vector alone.

Confocal microscopy studies in these MCF-7 cells stably transfected with the FLAG-CHK-pcDNA3neo construct demonstrated that CHK was localized in the cytosol fraction. However, upon heregulin stimulation, CHK was

translocated to the membrane. These results, taken together with the data on CHK-SH2 associating with ErbB-2, suggest that CHK is translocated from the cytosol to the membrane and associates with the ErbB-2 receptor upon ligand stimulation.

EXAMPLE 9: TUMOR DEVELOPMENT IN NUDE MICE

Initial studies have shown that CHK negatively regulates *src* activity and associates with ErbB-2 upon heregulin stimulation. Therefore, CHK might function as a negative regulator and might act to inhibit mitogenic signaling by c-src and ErbB-2. Interestingly, the proliferation rate of the MCF-7/CHK clone was reduced compared to the control MCF-7/neo clone or untransfected MCF-7 cells. Therefore, to evaluate the anti-transforming potential of CHK, tumor development was monitored in nude mice injected with MCF-7, MCF-7/neo and MCF-7/CHK cells, using standard laboratory techniques.

Tumor development in nude mice injected with MCF-7/CHK cells was significantly reduced (two out of fifteen tested) compared to tumor development in nude mice injected with control MCF-7 cells (15 of 15) or MCF-7/neo cells (12 of 15). These experiments suggest that overexpression of CHK can negatively regulate the growth of MCF-7 breast cancer cells in nude mice.

EXAMPLE 10: CHK OVEREXPRESSION AFFECTS S-PHASE ENTRY OF MCF-7 CELLS

A number of proto-oncogenes have been shown to affect cell cycle. Proto-oncogenes involved in the G_0/G_1 transition, such as myc and ras, are able to cooperate with cyclin D_1 in transforming cells. pp60src has been directly implicated in cell cycle regulation as well (Taylor, S.J. *et al.*, *Bioassays*, 18:9-11 (1996); Roche, S. *et al.*, *Science*, 269:1567-1569 (1995)). Since it has been demonstrated that CHK can regulate pp60src, it was investigated whether the level of CHK expression might modulate cell cycle kinetics using MCF-7 cells or

transfected MCF-7 cells that overexpressed CHK protein (i.e., MCF-7/CHK). Growth-arrested postconfluent MCF-7, MCF-7/neo or MCF-7/CHK cells were obtained by serum depletion for 4 days. Cells were stimulated by 10% serum and harvested at specific times. These analyses indicate a significant delay in the entry to S-phase of the CHK transfected MCF-7 cells compared to the controls. These results suggest that overexpression of CHK might have an effect on cell cycle.

EXAMPLE 11: SUPPRESSION OF CELL GROWTH BY CHK

To determine if CHK might affect the growth of MCF-7 cells, cells (10^3 cells/well) were spread in microtiter plates (96-wells) and the number of live cells was determined by using the MTT method (Rosenthal, A. *et al.*, *Cell*, 46:301-309 (1986)). The proliferation rate of the CHK-expressing cells (CHK-Flag-MCF-7) was significantly reduced ($p < 0.001$) compared to the control untransfected MCF-7 cells or cells transfected with vector alone (neo-1) (Figure 4A). Furthermore, when the cells expressing CHK were stimulated with HRG, a significant reduction in their proliferative response to HRG was observed (Figure 4B). These data suggest that CHK can reduce the proliferative activity of breast cancer cells and cause desensitization to the growth promoting effects of heregulin.

The anti-transforming potential of CHK was evaluated in MCF-7 clones by examining the ability of CHK-transfected cells to escape contact inhibition when grown on tissue culture plastic and to support anchorage-independent growth in soft agar. Cells (1×10^5 in 6-well dishes) were grown in medium containing 0.4% agar. After two weeks of growth, the colonies were visualized by staining with 0.33% iodonitrotetrazolium violet. As shown in Figure 4C, MCF-7 cells, as well as neo-1 cells, grew in culture plates to a higher density, displayed a tendency to pile up and acquired the ability to form colonies in soft agar. CHK-expressing cells, on the other hand, did not show anchorage-independent growth and the number of colonies

formed in soft agar decreased approximately 4-fold compared to control MCF-7 or neo-1 cells. Taken together, these results demonstrate that CHK expression is associated with anti-proliferative activity and can reduce the transformation ability of breast cancer cells.

EXAMPLE 12: EXPRESSION OF CHK USING THE VACCINE VIRUS/T7 RNA POLYMERASE HYBRID SYSTEM AND THE BACULOVIRUS SYSTEM

To analyze the interactions of CHK with ErbB-2, pp60src or other interacting molecules, a recombinant vaccinia virus was constructed to drive expression of CHK. CHK was inserted into a PTM-1 vaccinia recombinant plasmid under the control of the T7 RNA polymerase promoter. Recombinant viruses were selected, amplified and titered using standard techniques (Elroy-Stein, O. *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:6743-6747 (1990), Ausubel, F.M. *et al.*, *Current Protocols in Molecular Biology*, John Wiley & Son, Inc./Wiley Interscience, New York, NY (1992)). To demonstrate that recombinant viruses produce appropriately immunoreactive proteins, MCF-7 cells were co-infected with the CHK recombinant vaccinia virus and the T7 polymerase recombinant virus at 10 x MOI (multiplicity of infection) of each virus in 2.5% FCS DMEM. Cell lysates were run on SDS-PAGE and analyzed by immunoblotting with the anti-CHK antibodies. Expression of the 60 kDa immunoreactive CHK protein was demonstrated by immunoblotting with specific antibody. ^{35}S-labeling of MCF-7 cells co-infected with the CHK recombinant vaccinia virus indicated that CHK is a major protein being synthesized in these cells.

To characterize the biochemical and functional properties of CHK, CHK has also been expressed using the baculovirus system. For baculovirus expression, CHK cDNA was inserted into a pAcHLT-A™ vector (PharMingen), as directed by the manufacturer. Recombinant CHK baculovirus was used to infect Sf9 insect cells for 72 hours at 5 x MOI. Cell lysates were run on SDS-PAGE followed by Western blotting with anti-CHK antibody, or by protein staining of the gel with Coomassie Blue. Extracts of recombinant CHK

baculovirus derived from infected Sf9 cells were chromatographed on phosphotyrosine-Affi-gel, DEAE-Sephacel, and Mono S. Purified CHK was eluted from these columns as described in Flink, N.A. *et al.*, *J. Cell. Biochem.*, 55:389-397 (1994).

EXAMPLE 13: INVESTIGATION OF ErbB-2 SIGNALING MECHANISMS BY VACCINIA-DRIVEN OVEREXPRESSION OF CHK IN MCF-7 CELLS

To elucidate the involvement of CHK in the regulation of *src* kinase activity, CHK was overexpressed using the T7 polymerase-vaccinia expression system. MCF-7 cells were coinfected with either a CHK-vaccinia recombinant virus (CHK-vacc) and T7 polymerase virus (T7), or with the T7 virus alone as a control.

Approximately 5 x 10^5 cells/plate were seeded. One day later, the cells were infected with trypsinized recombinant viruses 10 x MOI for 1-2 hours in 2.5% FCS DMEM at 37°C. Next, 5 ml of 10% DMEM was added and the plates were incubated overnight. One day post-infection, the cells were starved for 4 hours in serum-free media, then stimulated with 10 nM heregulin (Zrihan-Licht, *S. et al.*, *J. Biol. Chem.*, 272:1856-1863 (1997)). Cell extracts were immunoprecipitated using *src* antibody, and the enzymatic activity of *src* was determined using Poly Glu/Tyr (4:1) as a substrate. In CHK-expressing cells, Poly Glu/Tyr phosphorylation was decreased about 4-fold compared to the control T7 infected cells upon stimulation with heregulin (Figure 5). Therefore, CHK expression resulted in a significant reduction in *src* kinase activity upon heregulin stimulation, indicating that CHK may regulate the ErbB-2 ctivated *src* kinases.

EXAMPLE 14: CHK PHOSPHORYLATION OF THE C-TERMINAL *src* PEPTIDE, ENOLASE, AND POLY GLU/TYR

In order to confirm the pp60src kinase as a substrate for CHK, immunoprecipitations of CHK and Csk from mouse brain were carried out. Mouse brain extracts were immunoprecipitated with either anti-CHK (murine Ctk), anti-

Csk (murine), or normal mouse serum. Washed immunoprecipitates were used to phosphorylate substrates in the presence of 25 mM MOPS, pH 7.4, 50 μM Na_3VO_4 5 mM $MnCl_2$, 0.5 mM DTT, 125 μM $\gamma[^{32}P]$ ATP. Substrates tested were C-terminal *src* peptide (▒), enolase (■), and Poly Glu/Tyr (□). Reactions were either terminated by the addition of SDS sample buffer (enolase, Poly Glu/Tyr) and run on SDS-PAGE, or terminated by pipetting onto P81 paper (src peptide) and washed extensively in 75 mM phosphoric acid. In vitro kinase assays of CHK and Csk immunoprecipitates showed that both kinases phosphorylated the C-terminal *scr* peptide, enolase and Poly Glu/Try to similar degrees (Figure 6).

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

What is claimed is:

1. A diagnostic kit comprising a reagent for detecting the expression of Csk Homologous Kinase in breast tissue (e.g. in human breast tissue).

2. An agent for detecting the expression of Csk Homologous Kinase (or a biologically active fragment, analog or derivative thereof) in breast tissue, for use in diagnosis (for example in the diagnosis of breast cancer, e.g. in humans).

3. Use of an agent for detecting the expression of Csk Homologous Kinase in breast tissue in the manufacture of a composition for use in diagnosis (for example the diagnosis of breast cancer, e.g. in humans).

4. A process for the manufacture of a diagnostic composition (for example for diagnosing breast cancer, e.g. in humans), characterized in the use, as an essential constituent of said composition, of an agent for detecting the expression of Csk Homologous Kinase in breast tissue.

5. An *ex vivo* (e.g. *in vitro*) method of detecting the presence of cancer in breast tissue (e.g. human breast tissue) comprising detecting the expression of Csk Homologous Kinase in breast tissue wherein the detection of the expression of Csk Homologous Kinase is indicative of cancer.

6. A pharmaceutical composition (for example a topical formulation, e.g. comprising liposomes) comprising:
   (a) Csk Homologous Kinase (or biologically active fragment, analog, variant or derivative thereof); or

(b) a nucleic acid construct containing a nucleic acid sequence encoding Csk Homologous Kinase (or a biologically active fragment, analog, variant or derivative thereof).

7. A liposome comprising:
(a) Csk Homologous Kinase (or biologically active fragment, analog, variant or derivative thereof); or
(b) a nucleic acid construct containing a nucleic acid sequence encoding Csk Homologous Kinase (or a biologically active fragment, analog, variant or derivative thereof).

8. Csk Homologous Kinase (or a biologically active fragment, analog, variant or derivative thereof) for use in therapy or prophylaxis.

9. 9. Use of:
(a) Csk Homologous Kinase (or a biologically active fragment, analog, variant or derivative thereof); or
(b) a nucleic acid construct containing a nucleic acid sequence encoding Csk Homologous Kinase (or a biologically active fragment, analog, variant or derivative thereof),
for the manufacture of a medicament for use in therapy or prophylaxis.

10. A process for the manufacture of an agent for therapy or prophylaxis, characterized in the use, as an essential constituent of said composition, of:
(a) Csk Homologous Kinase (or a biologically active fragment, analog, variant or derivative thereof); or
(b) a nucleic acid construct containing a nucleic acid sequence encoding Csk Homologous Kinase (or

a biologically active fragment, analog, variant or derivative thereof).

11. The invention of any one of Claims 8-10 wherein the therapy or prophylaxis comprises:

(a) the treatment or prevention of breast cancer (e.g. in humans); and/or

(b) inhibiting ErbB-2 mediated neoplastic cell growth; and/or

(c) the treatment or prevention of conditions in which negative regulation of ErbB-2 mediated mitogenic signaling is indicated; and/or

(d) the treatment or prevention of conditions associated with ErbB-2 overexpression; and/or

(e) negative regulation of ErbB-2 mediated mitogenic signaling.

FIG. 1A

FIG. 1B

FIG. 1C

FIG. 1D

FIG.2A

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   1 GGAGCAACTCGCTCCAAGTTGTGCAGCCGGGACCGCCTCGGGTGTGCAGCCGGCTCGCGAGGCCCTCCTGGGGGCGGGCGGGCGCGGCTCGG
  96 GGGCGCCCCCTGAGCAGAAAACAGGAAGAACCAGGCTCGGTCCATGTGGCACCCAGCTCCCTACCTCCTGTGCCAGCCGCCTGTGGCAGGC
                                                                                  MetAlaGlyArgGlySerLeuVal
 191 CATTCCCAGCGTCCCCGACTGTGACCACTTGCTCAGTGTGCCTCTCACCTGCCTCAGTTTCCTCTGGGGGCGATGGCGGGCGAGGCTCTCTGGT
          10                  20                  30                  40
       SerTrpArgAlaPheHisGlyCysAspSerAlaGluGluLeuProArgArgValSerProArgPheLeuArgAlaTrpHisProProProValSerAla
 286 TTCCTGGCGGGCATTTCACGGCTGTGATTCTGCTGAGGAACTTCCCCGGGTGAGCCCCGCGCTTCCTCCGAGCCTGGCACCCCCCCTCCCGTCTCAG
                      50                  60                  70
       ArgMetProThrArgArgTrpAlaProGlyThrGlnCysIleThrLysCysGluHisThrArgProLysProGlyGluLeuAlaPheArgLys
 381 CCAGGATGCCAACGAGGCGCTGGGCCCCGGGCACCCAGTGTATCACCAAATGCGAGCACACCCGCCCCAAGCCAGGGGAGCTGGCCTTCCGCAAG
                      90                  100                 110
       GlyAspValValThrIleLeuGluAlaCysGluAsnLysSerTrpTyrArgValLysHisHisThrSerGlyGlnGluGlyLeuLeuAlaAlaGly
 476 GGCGACGTGGTCACCATCCTGGAGGCCTGCGAGAACAAGAGCTGGTACCGCGTCAAGCACCACACCAGTGGACAGGAGGGCTGCTGGCAGCTGG
                      110                 120                 130
       AlaLeuArgAspGlyGluAlaLeuSerAlaProProLysLeuSerLeuMetProTrpPheHisGlyLysIleSerGlyGlnGluAlaValGlnGln
 571 GGCGCTGCGGGACGGGGAGGCCCTCTCCGCAGACCCCAAGCTCAGCCTCATGCCGTGGTTCCACGGGAAGATCTCGGGCCAGGAGGCTGTCCAGC
                      140                 150                 160
       LeuGlnProProGluAspGlyLeuPheLeuValArgGluSerAlaArgHisProGlyAspTyrValLeuCysValSerPheGlyArgAspVal
 666 AGCTGCAGCCTCCCCGAGGATGGGCTGTTCCTGGTGCGGGAGTCCGCGCGCCACCCCGGCGACTACGTCCTGTGTGCGTTTGGCCGCGACGTC
                      170                 180
       IleHisTyrArgValLeuHisArgAspGlyHisLeuThrIleAspGluAlaValPhePheCysAsnLeuMetAspMetValGluHisTyrSerLys
 761 ATCCACTACCGCGTGCTGCACCGCGACGGCCACCTCACAATCGATGAGGCCGTTCTTCTTCTGCAACCTCATGGACATGGTGGAGCATTACAGCAA
                      200                 210                 220                 230
       AspLysGlyAlaIleCysThrLysLeuValArgProLysArgTyrSerHisGlyThrLysSerAlaGluGluLeuAlaArgAlaGlyTrpLeuLeu
 856 GGACAAGGGCGCTATCTGCACCAAGCTGGTGAGACCAAAGCGGAAACACGGGACCAAGTCGGCCGAGGAGGAGCTGGCCAGGGCGGGCTGGTTAC
                      240                 250                 260
       AsnLeuGlnHisLeuThrLeuGlyAlaGlnIleGlyGluGlyGluPheGlyAlaValLeuGlnGlyGluTyrLeuGlnLysValAlaValAlaVal
 951 TGAACCTGCAGCATTTGACATTGGGAGCACAGATCGGAGAGGGAGAGTTTGGAGCTGTCCTGCAGGGTGAGTACCTGGGGCAAAAGGTGGCCGTG
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FIG.2B

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                                270                             280                             290
      LysAsnIleLysCysAspValThrAlaGlnAlaPheLeuAspGluThrAlaValMetThrLysMetGlnHisGluAsnLeuValArgLeuLeuGly
1046  AAGAATATCAAGTGTGATGTGACAGCCCAGGCCTTCCTGGACGAGACGGCCGTCATGACGAAGATGCAACACGAGAACCTGGTGCGTCTCCTGGG
                          300                             310                             320
      ValIleLeuHisGlnGlyLeuTyrIleValMetGluHisValSerLysGlyAsnLeuValAsnPheLeuArgThrArgGlyArgAlaLeuValAsn
1141  CGTGATCCTGCACCAGGGGCTGTACATTGTCATGGAGCACGTGAGCAAGGGCAACCTGGTGAACTTTCTGCGGACCCGGGGTCGAGCCCTCGTGA
                    330                             340                             350
      ThrAlaGlnLeuLeuGlnPheSerLeuHisValAlaGluGlyMetGluTyrLeuGluSerLysLysLeuValHisArgAspLeuAlaAlaArg
1236  ACACCGCTCAGCTCCTGCAGTTTTCTCTGCACGTGGCCGAGGGCATGGAGTACCTGGAGAGCAAGAAGCTTGTGCACCGCGACCTGGCCGCCCGC
                360                             370                             380
      AsnIleLeuValSerGluAspLeuValAlaLysValSerAspPheGlyLeuAlaLysAlaGluArgLysGlyLeuAspSerArgLeuProVal
1331  AACATCCTGGTCTCAGAGGACCTGGTGGCCAAAGGTCAGCGACTTTGGCCTGGCCAAAGCCGAGCGGAAGGGGCTAGACTCAAGCCGGCTGCCCGT
          390                             400                             410                             420
      LysTrpThrAlaProGluAlaLeuLysHisGlyPheThrSerLysSerAspValTrpSerPheGlyValLeuLeuTrpGluValPheSerTyrGly
1426  CAAGTGGACGGCGCCCGAGGCTCTCAAACACGGGTTCACCAGCAAGTCGGATGTCTGGAGTTTTGGGGTGCTGCTCTGGGAGGTCTTCTCATATG
                          430                             440                             450
      ArgAlaProTyrProLysMetSerLeuLysGluValSerGluAlaValGluLysGlyTyrArgMetGluProProGluGlyCysProGlyPro
1521  GACGGGCTCCGTACCCTAAAATGTCACTGAAAGAGGTGTCGGAGGCCGTGGAGAAGGGGTACCGCATGGAACCCCCCGAGGGCTGTCCAGGCCCC
                    460                             470                             480
      ValHisValLeuMetSerSerCysTrpGluAlaGluProProAlaGlyHisProSerAlaAsnTrpProArgSerTrpProGlySerTyrAlaVal
1616  GTGCACGTCCTCATGAGCAGCTGCTGGGAGGCAGAGCCGCCCGCCGGCCACCCTTCCGCAAACTGGCCGAGAAGCTGGCCCGGGAGCTACGCAGT
                490                             500                             510
      GlnValProGlnProProSerGlnGlyArgThrProThrValHisLeuAlaProLysProGlyAlaLeuThrProProGlyGlyProTrpProGln
1711  GCAGGTGCCCCAGCCTCCGTCTCAGGGCAGGACGCCGACGGTCCACCTCGCCCCGAAGCCAGGAGCCCTGACCCCACCCGGTGGCCCTTGGCCCC
          520
      ArgThrGluArgValGluSerAlaAlaTrpGlyHis
1806  AGAGGACCGAGAGAGTGGAGAGTGCGGCGTGGGGGCACTGACCAGGCCCAAGGAGGGTCCAGGCGGGCAAGTCATCCTCCTGGTGCCCACAGCAG
1901  GGGCTGGCCCACGTAGGGGGCTCTGGGCGGCCCGTGGACACCCCAGACCTGCGAAGGATGATCGCCCGATAAAGACGGATTCTAAGG
```

FIG. 3

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1    PTK 1/3 PRIMERS      21                  41
5' ggatccattcacagagacctagcagcacgcaacaatcctggtctcagaggacctggtaacc
     G  S  I  H  R  D  L  A  A  R  N  I  L  V  S  E  D  L  V  T

61                        81                  101
aaggtcagcgactttggcctggccaaagccgagcggaaggggctagactcaagccggctg
     K  V  S  D  F  G  L  A  K  A  E  R  K  G  L  D  S  S  R  L

121      PTKKW  PRIMER  141
cccgtcaaatggatggctcccgaattc 3'
     P  V  K  W  M  A  P  E  F
```

FIG. 4A

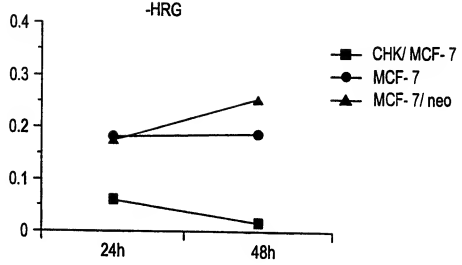


FIG. 4B

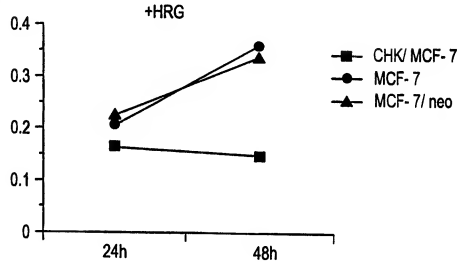


FIG. 4C

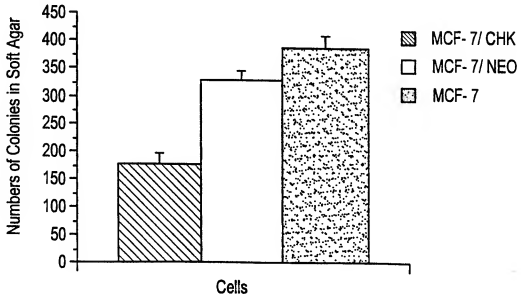


FIG. 5

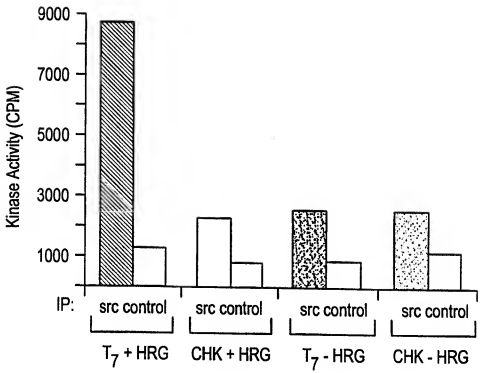
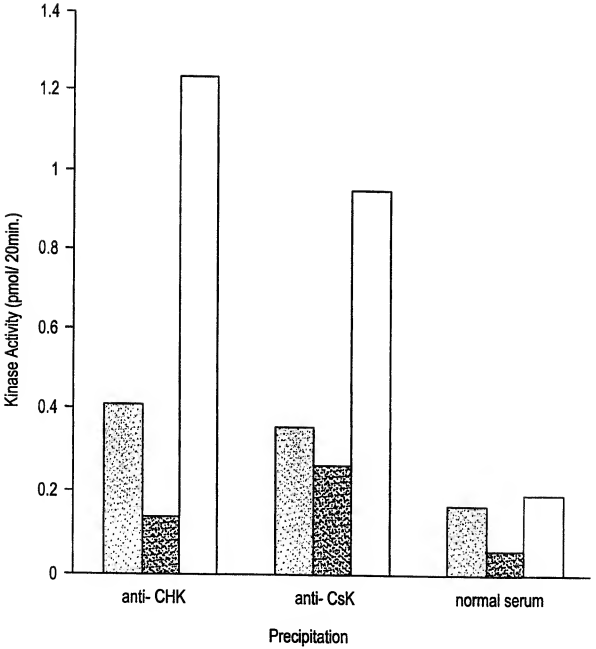

9000
7000
5000
3000
1000
Kinase Activity (CPM)
IP:
src control
src control
src control
src control
T7 + HRG
CHK + HRG
T7 - HRG
CHK - HRG

FIG. 6


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Kinase Activity (pmol/ 20min.)
anti- CHK
anti- CsK
normal serum
Precipitation

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/US 98/00420

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 G01N33/50 C12N9/12 A61K38/45 A61K48/00
A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N G01N A61K

Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ZRIHAN-LICHT S ET AL: "Association of csk-homologous kinase (CHK) (formerly MATK) with HER-2/ErbB-2 in breast cancer cells." J BIOL CHEM, JAN 17 1997, 272 (3) P1856-63, UNITED STATES, XP002066240 see abstract ---	1-11
T	ZRIHAN-LICHT S ET AL: "Csk homologous kinase, a novel signaling molecule, directly associates with the activated ErbB-2 receptor in breast cancer cells and inhibits their proliferation." J BIOL CHEM, FEB 13 1998, 273 (7) P4065-72, UNITED STATES, XP002066241 -----	

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
28 May 1998	16/06/1998

Name and mailing address of the ISA	Authorized officer
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